Proton Nuclear Magnetic Resonance Study of Cobrotoxin†

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ABSTRACT: Cobrotoxin (M_r 6949), which binds tightly to the acetylcholine receptors, contains no phenylalanines and only two histidines, two tyrosines, and one tryptophan that result in well-resolved aromatic proton resonances in D_2O at 360 MHz. His-32, Tyr-25, and the Trp are essential for toxicity and may interact with the acetylcholine receptor. We assign two titratable resonances (pK_a = 5.1) at δ = 9.0 and 7.5 ppm at pH 2.5 and at 7.7 and 7.1 ppm at pH 9.5 to the C-2 and C-4 ring protons, respectively, of His-4. Two other titratable resonances (pK_a = 5.7) at δ = 8.8 and 6.9 ppm at pH 2.5 and at 7.8 and 6.7 ppm at pH 9.5 are assigned to the C-2 and C-4 ring protons of His-32, respectively. The differences in δ values of the two histidines reflect chemically different microenvironments while their low pK_a values could arise from nearby positive charges. A methyl resonance gradually shifts upfield to $\delta \sim 0.4$ ppm as His-4 is deprotonated and is tentatively assigned to the methyl group of Thr-14 or Thr-15 which, from published X-ray studies of neurotoxins, are located in the vicinity of His-4. Further, we have identified the aromatic resonances of the invariant tryptophan and individual tyrosines and the methyl resonance of one of the two isoleucines in the molecule. Several broad nontitrating resonances of labile protons which disappear at pH >9 may arise from amide groups of the β sheet in cobrotoxin.

Neurotoxins from various snake venoms have been extensively investigated (Tu, 1973). Cobrotoxin from Taiwan cobra (*Naja naja atra*) is one of these protein neurotoxins which bind with high affinity to the nicotinic acetylcholine receptor of the postsynaptic membrane, thus blocking neuromuscular transmission across cholinergic synapse (Lee, 1972). Cobrotoxin consists of 62 amino acids (M_r 6949) that make up a single polypeptide chain of known amino acid sequence containing four disulfide bonds (Yang et al., 1969, 1970; Yang, 1974). Previous studies on structure–toxicity relationships of cobrotoxin have indicated that Trp-29 (Chang & Yang, 1973), Tyr-25 (Chang et al., 1971), His-32 (Huang et al., 1972), Arg-30, and Arg-33 (Yang et al., 1974) are essential for full activity of the toxin. Superimposing the primary structure of cobrotoxin onto the known three-dimensional structures of Philippines sea snake toxin *b* (Tsernoglou & Petsko, 1976, 1977) and erabutoxin *b* (Low et al., 1976) shows that Tyr-25, Trp-29, His-32, Arg-30 and Arg-33 are located in the protruding loop with His-32 at the tip of the loop that may interact with the acetylcholine receptor (Figure 1).

Cobrotoxin is particularly amenable to ^{1}H NMR studies owing to its small size, high solubility, stability, and the presence in it of two titratable histidines, two tyrosines, one tryptophan, and no phenylalanines which may be expected to result in a simplified aromatic region of the protein spectrum (Arseniev et al., 1976). The present investigation represents the initial stage of an NMR study of the conformation and dynamics of cobrotoxin in solution and the mechanism of its interaction with acetylcholine receptors. Prerequisite to such a program is the identification of spectral resonances with specific nuclei of the neurotoxin. In this communication, we present the assignments of resonances in the entire aromatic region of the ^{1}H NMR spectrum of cobrotoxin. These assignments are based on the following three criteria: (1) the titratability and pH titration behavior of the resonances, (2) a comparison of the absolute chemical shifts and coupling constants of proton resonances of cobrotoxin with those of isolated amino acids, and (3) the known amino acid sequence and three-dimensional structure of a similar neurotoxin. A preliminary account of this work was presented at the joint Biophysical Society/American Physical Society annual meeting in Washington, DC, March 27–29, 1978, and an abstract of the work has been published (Fung et al., 1978).

Materials and Methods

Cobrotoxin was prepared from Taiwan cobra (*Naja naja atra*) venom as previously described (Yang, 1965). Proton NMR spectra were recorded in 5-mm o.d. NMR tubes (Wilmad Glass Co., Buena, NJ) at 100 MHz on a Varian XL-100 NMR spectrometer at the Institute for Cancer Research and at 360 MHz on a Bruker HX360 NMR spectrometer at the Middle Atlantic Regional NMR Facility located at the University of Pennsylvania. Samples for NMR were deuterated by dissolving the previously lyophilized sample of essentially salt-free cobrotoxin in D_2O. Field-frequency stabilization was accomplished by locking on the ^{2}H signal of the solvent. The pH was adjusted by the addition of 100 mM DCl or 100 mM NaOD (Merck Sharp & Dohme of Canada) as desired. The reported pH values are readings on Radiometer Model 26 pH meter and were not corrected for possible deuterium isotope effects. The concentration of cobrotoxin used was in the range 3–7 mM. All spectra were obtained in the Fourier transform mode time-averaging 64 transients of free induction signal at 360 MHz or by time-averaging 512 transients at 100 MHz. The pH titration curves of the histidine ring protons were obtained at 100 MHz. To avoid the dynamic range problem in recording proton spectra (Redfield & Gupta, 1971) of cobrotoxin, the unwanted proton signal from residual HDO was kept suppressed by saturating water spins with radio frequency energy using the Varian spin decoupler (Gupta et al., 1976). All experiments were done at 23 °C and the chemical shifts are referenced to external DSS standard.

Results and Discussion

The 360-MHz ^{1}H NMR spectra of cobrotoxin in a D_2O solution of the protein at several pH values in the range 2.5–12 are shown in Figures 2 and 3. Several exchangeable resonances are observed in the chemical shift range ~7–10 ppm

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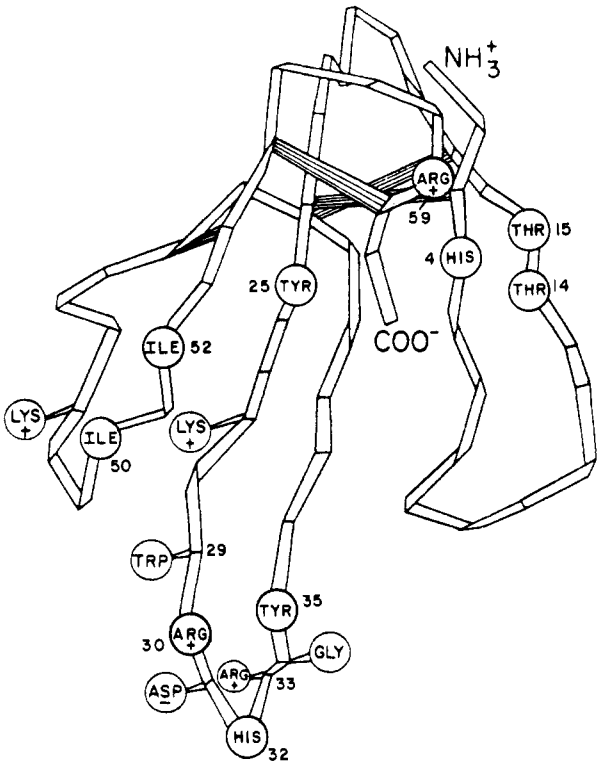

FIGURE 1: Superimposition of the primary structure of cobrotoxin onto the known three-dimensional structure of Philippines sea snake toxin *b* (Tsernoglou & Petsko, 1977).

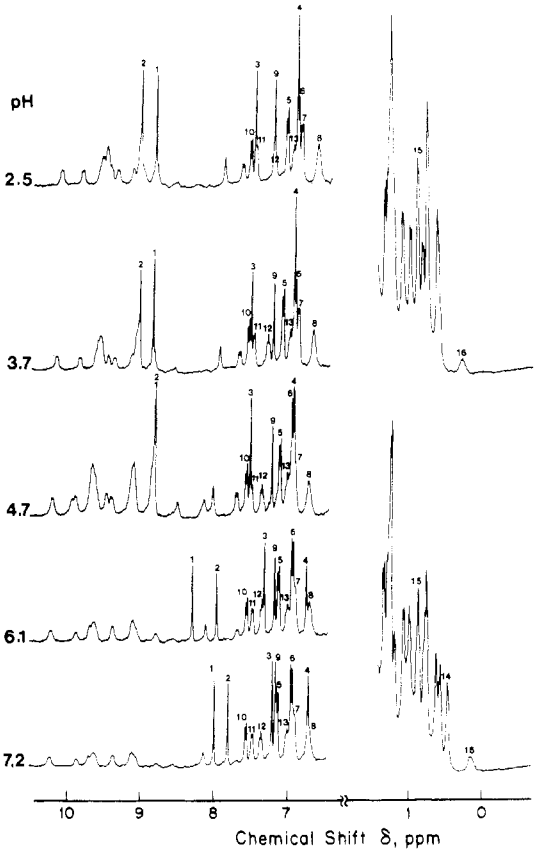

FIGURE 2: 360-MHz proton NMR spectra of a 6 mM cobrotoxin solution at several pH values in the range 2.5–7.2. Chemical shifts are referenced to external DSS. Resonances 1–4 are assigned to His aromatic protons, 5–8 to Tyr ring protons, 9–13 to the Trp aromatic protons, 14 to a Thr methyl group, and 15 to the methyl group of one of the two isoleucines.

downfield from DSS[1] at pH values below neutrality and must be amide protons (exchange time ≥6 h at pH ≤7) which are deuterated in ≤5 min at pH ≥9.5 (Figure 3). The presence of several resonances of unexchanged amide protons in the NMR spectra of cobrotoxin in D_2O is understandable in the light of the recently published X-ray crystallographic structure of similar neurotoxins (Tsernoglou & Petsko, 1977; Low et al., 1976) which showed that the molecule lacks the α-helix structure but consists of β turns and antiparallel β-pleated sheet, resulting in slowly exchanging hydrogen-bonded amide protons. The chemical shifts of four sharp, unsplit resonances of equal intensity at 9.0, 8.8, 7.5, and 6.9 ppm at pH 2.5 titrate upfield with increasing pH in the pH range 3–7 and, on the basis of their titratability and pK_a values, are assigned to the four ring protons of the two histidines. A comparison of the area under the histidine peaks on expanded spectra with the total spectral absorption area under all nonexchangeable resonances in the aromatic region from 6.5 to 9 ppm indicates the presence of resonances of 17 ± 1 nuclei, which shows that we can observe the resonances of all seventeen nonexchangeable aromatic protons in the molecule. For this comparison of absorption areas under peaks, fully relaxed spectra corresponding to a pulse recycle time of 16 s (≥7 T_1) were used. Some resonances are also observed in the region between HDO and 6.5 ppm. The origin of these resonances is not entirely clear at present, but it is likely that they represent carbon-bound methine protons of constituent amino acids, which are shifted somewhat downfield relative to their positions in isolated amino acids by their interactions with other groups in the protein.

Titration and Assignment of Resonances Corresponding to His-4 and His-32. The two sharp single proton resonances at 9.0 and 8.8 ppm at pH 2.5 move upfield with increasing pH (Figure 2) to 7.7 and 7.8 ppm at pH 8.0 but become invariant with pH above pH 8. The titration curves characterizing the pH dependence of their chemical shifts are shown in Figure 4. Resonance 2 which starts at 9.0 ppm at pH 2.5, ~0.2 ppm downfield with respect to resonance 1, moves upfield with increasing pH more rapidly compared to resonance 1 and crosses resonance 1 in the spectrum at pH 4.7 (Figures 2 and 4). The titration data points in Figure 4 (open and closed circles correspond to resonances 2 and 1, respectively) were fitted to the theoretical Henderson–Hasselbalch equation for ionization of a protonated amino acid. The solid lines through the data points in Figure 4 are theoretical curves and yield pK_a values of 5.7 ± 0.1 and 5.1 ± 0.1 for peaks 1 and 2, respectively. The sharpness and titratability of these resonances, their absolute chemical shifts, the magnitude of changes in their chemical shifts with pH (~1 ppm), and the measured pK_a values establish the origin of these resonances from the C-2 ring protons of the two histidines. Histidine resonances in proteins are well known to titrate upfield with increasing pH with a pK_a near neutrality as the His rings lose part of their aromaticity by deprotonation (Cohen, 1969; McDonald & Phillips, 1969; Meadows et al., 1967; Roberts & Jardetzky, 1970; Meadows & Jardetzky, 1968; Cohen et al., 1972; Markley, 1973; Sachs et al., 1971; Schechter et al., 1972; Shrager et al., 1972). Two other titratable single sharp resonances (3 and 4) are observed at 7.5 and 6.9 ppm at pH 2.5 (Figure 2). These resonances move to 7.1 and 6.7 ppm at pH ≥8 (Figure 3), and their pH-dependent chemical shifts follow titration curves parallel to those of the C-2 protons in Figure 4. These resonances (3 and 4) are assigned to the C-4 protons of the two histidines on the

[1] Abbreviation used: DDS, 4,4-dimethyl-4-silapentane-1-sulfonate.

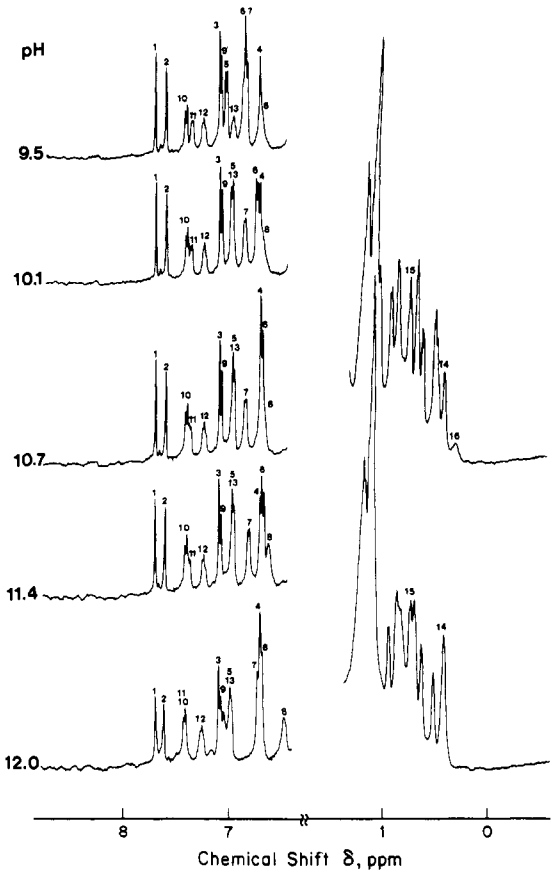

FIGURE 3: 360-MHz proton NMR spectra of a 6 mM cobrotoxin solution at several pH values in the range 9.5–12. Assignments of numbered resonances are discussed in Figure 1 and the text. Chemical shifts are referenced to external DSS.

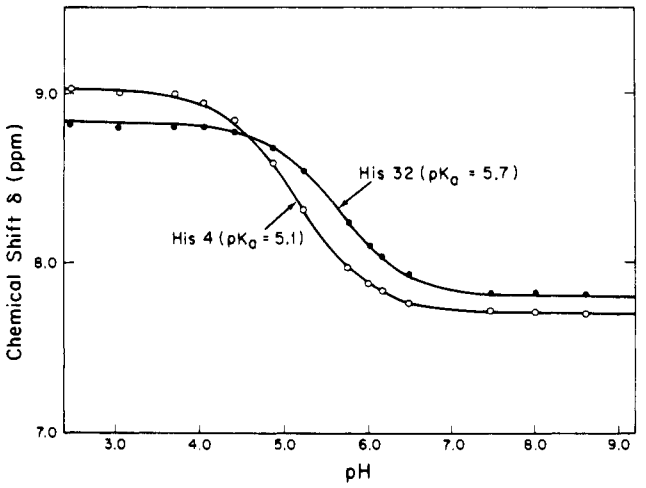

FIGURE 4: Data showing pH titration of histidine C-2 protons in cobrotoxin. Solid lines represent theoretical curves obtained using the Henderson–Hasselbalch relationship and pK_a values of 5.1 and 5.7.

basis of their sharpness, absolute chemical shifts, titratability, pH titration behavior, and the magnitude of changes in their chemical shifts with pH. The observation of sharp histidine ring proton resonances indicates that both histidine rings in cobrotoxin are free to rotate since immobilized histidines are often broadened beyond detection in proteins (Bradbury & Wilairat, 1967). The differences in the absolute chemical shifts of the corresponding ring protons of the two histidines reflect differing microenvironments due to tertiary folding of the protein (Cohen, 1969; Sachs et al., 1971; Schechter et al., 1972; Shrager et al., 1972). The low pK_a values of both histidines (pK_a = 5.1 and 5.7) relative to isolated histidine (pK_a ~ 6.5) in solution reflect the altered electrostatic environments of both of them in the protein while the ~0.6-unit difference in their pK_a values can be understood in terms of the presence of the negatively charged aspartic acid residue 31 near histidine-32, which would raise its pK_a above that of His-4. On this basis, resonances 2 and 3 are assignable to the C-2 and C-4 ring protons of His-4 and the remaining two (1 and 4) to His-32. The observation of a single sharp resonance from protonated and unprotonated forms of the histidines in their partly ionized states reflects rapid interconversion ($\geq 10^3$ s^{-1}) of the two forms. Rapid protonation and deprotonation of the histidines indicate free accessibility of both histidines to solvent water molecules, although only one of them (His-32) is readily accessible to chemical modifications (Huang et al., 1972).

Assignment of Tyrosine Aromatic Protons. Of the remaining resonances, the ones corresponding to the aromatic protons of the two tyrosines can be separated from those of the single tryptophan by a study of their pH dependencies at pH values above 9 since the phenolic proton of a tyrosine side chain titrates with a pK_a ~ 10, altering the positions of the aromatic resonances of the tyrosine ring (Karplus et al., 1973; Roberts & Jardetzky, 1970). Two resonance doublets (5 and 6), each arising from two equivalent protons, titrate upfield with increasing pH (pK_a ~ 9.7) and are assigned to Tyr-35 (Figure 3). It is known from the spectrophotometric titrations of cobrotoxin that only one of the two tyrosyl side chains titrates freely with a normal apparent pK_a of 9.65 and that the other tyrosine is buried and ionizes only above pH 11.3 (Chang et al., 1971). The titratable tyrosine with normal pK_a has been identified with Tyr-35 on the basis of chemical modification studies. Thus the side chain of Tyr-35 is more readily accessible to nitration. X-ray crystallographic studies of a similar neurotoxin (Tsernoglou & Petsko, 1977) also suggest that Tyr-25 would be completely buried in the hydrophobic interior of the molecule. At pH ~11, the two doublets, 7 and 8, of two protons each, corresponding to Tyr-25 also begin to titrate, but irreversible changes in the NMR spectrum occur at pH >11.5, and, therefore, complete pH titrations of these resonances could not be observed (Figure 3). On the basis of their intensity, chemical shifts, pH titration behavior, and doublet character, therefore, resonance multiplets 5,6 and 7,8 are assigned to the 2,6 and 3,5 protons of Tyr-35 and Tyr-25, respectively.

Assignment of Trp-29 Resonances. The single sharp nontitrating resonance 9 is assigned to Trp-29 C-2 proton on the basis of its chemical shift (~7.2 ppm) and the absence on it of any spin–spin splittings (Robert & Jardetzky, 1970; McDonald & Phillips, 1969). The remaining two nontitrating one-proton doublets (10 and 11) and two one-proton triplets (12 and 13), one of which is clearly resolved in the spectrum only at pH ~9.5 (Figure 3), are assigned to the Trp protons 4,7 and 5,6, respectively. Further work including careful spin–spin decoupling experiments may be needed to distinguish between the two possible assignments.

Assignment of Resonances Corresponding to an Isoleucine Methyl and a Possible Threonine Methyl Group. A three-proton doublet resonance (14) shifts upfield to δ = 0.4 ppm as the histidines deprotonate, suggesting interaction of an amino acid residue containing a methyl group with one of the two histidines. With histidines in the protonated state, this resonance appears to be located at δ = 0.6 ppm. The pH dependence of the upfield shift of this resonance correlates better with the titration of His-4 (pK_a = 5.1) than with the

titration of His-32 (pK_a = 5.7). From the X-ray structure of a similar neurotoxin (erabutoxin *b*), one would place a threonine (Thr-14 or Thr-15) spatially close to His-4 in cobrotoxin (Figure 1), suggesting the possibility of some electrostatic interaction between the two residues. This electrostatic interaction may also be responsible for the larger change (Δ) between the chemical shifts of the protonated and unprotonated forms of His-4 C-2 proton (Δ = 1.3 ppm) compared to that of His-32 (Δ = 1 ppm). Another methyl resonance (15) with partly resolvable 1:2:1 triplet structure, due to spin–spin coupling to an adjacent methylene group, at 0.9 ppm downfield from DSS must arise from the methyl group of one of the two isoleucines in the protein since isoleucines are the only methyl-containing amino acid residues of the protein capable of giving rise to the observed triplet structure on the methyl resonance. All other methyl resonances are expected to be doublets or singlets. A broad single-proton resonance (16) located at the high field end of the NMR spectrum at 0.2 ppm downfield from DSS at pH 7 yet remains to be identified. It is essentially unaffected by histidine titrations but is gradually displaced downfield to δ = 0.4 ppm at pH 12 and may be an additional marker for the native protein conformation.

Conclusion

In conclusion, we have obtained and analyzed well-resolved proton NMR spectra of cobrotoxin at 360 MHz showing spin–spin couplings. Resonances corresponding to His-4, His-32, Tyr-35, and Tyr-25 have been assigned on the basis of their pH dependencies. The pH independence of the remaining resonances in the aromatic region and their observed multiplet patterns were used to assign them to Trp-29. His-32 and His-4 titrate with pK_a values of 5.7 and 5.1, respectively, consistent with their location in different chemical environments and with the presence of negatively charged aspartic acid residue 31 near His-32. The upfield shift of a doublet methyl resonance to δ = 0.4 ppm as His-4 deprotonates suggests some electrostatic interaction of His-4 with Thr-14 or Thr-15.

Our assignment of the entire aromatic region of the proton NMR spectrum of cobrotoxin, including amino acid residues essential for toxicity, provides a new means for studying the interaction of a neurotoxin with its intact, isolated, or chemically modified receptors. Such studies are continuing.

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References

Arseniev, A. S., Balashova, T. A., Utkin, Y. N., Tsetlin, V. I., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1976) *Eur. J. Biochem. 71*, 595–606.

Bradbury, J. H., & Wilairat, P. (1967) *Biochem. Biophys. Res. Commun. 29*, 84–89.

Chang, C. C., & Yang, C. C. (1973) *Biochim. Biophys. Acta 295*, 595–604.

Chang, C. C., Yang, C. C., Hamaguchi, K., Nakai, K., & Hayashi, K. (1971) *Biochim. Biophys. Acta 236*, 164–173.

Cohen, J. S. (1969) *Nature (London) 223*, 43–46.

Cohen, J. S., Yim, C. T., Kandel, M., Gornall, A. G., Kandel, S. I., & Freedman, M. H. (1972) *Biochemistry 11*, 327–334.

Fung, C. H., Chang, C. C., & Gupta, R. K. (1978) *Biophys. J. 21*, 199a.

Gupta, R. K., Fung, C. H., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2421–2430.

Huang, J. S., Liu, S. S., Ling, K. H., Chang, C. C., & Yang, C. C. (1972) *T'ai-wan I Hsueh Hui Tsa Chih 71*, 383–388.

Karplus, S., Snyder, G. H., & Sykes, B. D. (1973) *Biochemistry 12*, 1323–1329.

Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265–286.

Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991–2994.

Markley, J. L. (1973) *Biochemistry 12*, 2245–2250.

McDonald, C. C., & Phillips, W. D. (1969) *J. Am. Chem. Soc. 91*, 1513–1521.

Meadows, D. H., & Jardetzky, O. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 406–413.

Meadows, D. H., Markley, J. L., Cohen, J. S., & Jardetzky, O. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1307–1313.

Redfield, A. G., & Gupta, R. K. (1971) *Adv. Magn. Reson. 5*, 81–115.

Roberts, G. C. K., & Jardetzky, O. (1970) *Adv. Protein Chem. 24*, 447–545.

Sachs, D. H., Schechter, A. N., & Cohen, J. S. (1971) *J. Biol. Chem. 246*, 6576–6580.

Schechter, A. N., Sachs, D. H., Heller, S. R., Shrager, R. I., & Cohen, J. S. (1972) *J. Mol. Biol. 71*, 39–48.

Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., & Schechter, A. N. (1972) *Biochemistry 11*, 541–547.

Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1–4.

Tsernoglou, D., & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 971–974.

Tu, A. T. (1973) *Annu. Rev. Biochem. 42*, 235–258.

Yang, C. C. (1965) *J. Biol. Chem. 240*, 1616–1618.

Yang, C. C. (1974) *Toxicon 12*, 1–43.

Yang, C. C., Yang, H. J., & Huang, J. S. (1969) *Biochim. Biophys. Acta 188*, 65–77.

Yang, C. C., Yang, H. J., & Chiu, R. H. C. (1970) *Biochim. Biophys. Acta 214*, 355–363.

Yang, C. C., Chang, C. C., & Liou, I. F. (1974) *Biochim. Biophys. Acta 365*, 1–14.